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The Low-Molecular-Weight SH-Protease Inhibitor in Rat Skin is Epidermal

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The epidermis and dermis of rat skin were separated and the presence of the high-molecular-weight SH-protease inhibitor I_1 and the low-molecular-weight inhibitor I_2 in both was studied. Gel filtrations of the extracts revealed that 97% of the epidermal inhibitor activity was due to I_2 and 89% of the dermal activity to I_1. The presence of I_2 mainly in the epidermis extract was confirmed by immunodiffusion of specific rabbit anti-I_2 serum against purified I_2, epidermis and dermis extracts, and rat serum. Most of the immunoreactive protein was seen in the epidermis extract, traces in the dermis extract and none in the rat serum. I_2 was localized in rat skin by indirect immunofluorescence using rabbit anti-I_2 serum and fluorescein isothiocyanate conjugate of goat anti-rabbit immunoglobulins. Intense fluorescence, much brighter than in the controls treated with rabbit non-immune serum, was seen in the epidermis, being most pronounced in the cytoplasms of cells in the granular layer. The weak fluorescence of the hair follicles, sebaceous glands, connective tissue cells and fibres was unspecific and was also seen in the controls. In view of its epidermal location, the name epidermal SH-protease inhibitor is suggested for I_2.

Rat skin contains 2 inhibitors of SH-proteases, I_1 and I_2 [1,2]. I_1 has a molecular weight of 74,000 and inhibits papain, ficin, and bromelain, and to a minor extent rat skin benzoylarginine-2-naphthylamide hydrolase and cathepsin C. I_2 has a molecular weight of 13,400 and inhibits all the SH-proteases tested. Neither I_1 nor I_2 inhibits cathepsin D, trypsin, or chymotrypsin. I_1 is present in various rat tissues and in serum, and it has been suggested that I_1 in the rat skin is derived from serum [2]. On the other hand, I_2 is most abundant in the skin of the rat and other species and only small amounts are found in other tissues. We present evidence here for the location of I_2 in the epidermis of rat skin.

MATERIALS AND METHODS

I_2 was prepared by the method of Järvinen [2] and its activity assayed by measuring its effect on the activity of papain [2]. The unit of inhibitor activity was defined as milligrams of papain (E. Merck A.F., für biochemische Zwecke) inhibited by 1 ml of the inhibitor (U/ml) or by 1 mg of the inhibitor (U/mg). Proteins were assayed by the Lowry et al method [3]. Rabbit antiserum against I_2 was prepared and immunodiffusion performed as described earlier [2]. The immunoglobulin fraction of the antiserum was prepared as described by Harboe and Ingild [4].

Separation of the dermis and epidermis. The dorsal skin of a male Wistar rat (320 gm) was shaved with an electrical clipper and the residual hair removed using Veet cream (Reckit & Colman, Ltd, England). The rat was washed thoroughly with warm water and then was killed the following day, the skin being removed and the muscle layer peeled off with a blunt knife. The skin (weight 8 gm) was immersed in 1 M ammonia for 15 min and the epidermis scraped off with a blunt knife [5]. The dermis was minced with scissors and suspended in 40 ml of 10 mM phosphate buffer, pH 6, with 1% KCl. The pH was adjusted to 6.0 with 2 M HCl and an extract made as described earlier [2]. The epidermis was then suspended in the buffer solution and extracted in the same manner. The purity of the epidermis and dermis was monitored from histological preparations stained with hematoxylin-eosin.

Gel chromatography

A Sephadex G-50 column of dimensions 2.6 × 90 cm was equilibrated with 10 mM sodium phosphate buffer, pH 7.2, with 8.5 gm/l NaCl (PBS). Samples of 10 ml were applied at a flow rate of 40 ml/hr and fractions of 5.5 ml collected.

Immunofluorescence

The rat hair was removed as described above. Small pieces of skin (5 × 15 mm) were immersed in absolute ethanol at −60°C and dehydrated for 2 days at −60°C and then for 2 hr at room temperature. The pieces were then embedded in paraffin and cut into 6 μm sections. The sections were stretched on the surface of water for 10 sec at 37°C, transferred to albuminized microscope slides and dried at 37°C for 2 hr, deparaffinized in xylene and air dried. The sections were covered with various dilutions of anti-I_2 serum (rabbit) and incubated for 30 min in a moist chamber at room temperature, washed in circulating PBS for 30 min and then incubated for 30 min with fluorescein isothiocyanate conjugate of goat anti-rabbit immunoglobulin (Nordic Immunological Laboratories, batch No. 27-175), diluted 1/10 with 0.9% NaCl. Unadsorbed conjugate was removed by washing with PBS for 30 min at room temperature and the sections were mounted with phosphate buffered glycerol (one part of PBS + 9 parts of glycerol). Sections treated with rabbit non-immune serum instead of anti-I_2 serum served as controls. The anti-I_2 serum was adsorbed with the antigen as follows: 3 ml of I_2 (0.06 mg) was added to 1 ml of anti-I_2 serum, the mixture was allowed to stand for 30 min, and the precipitate was removed by centrifugation. The slides were examined with a Leitz SM-lux microscope equipped with an Osram HBO-200 lamp, Ploem illuminator No. 3, excitation filters BG-12 (3 mm) and KP 500, and emission filter K 510. A Leitz Orthomat camera with Kodak High Speed Ektachrome daylight film was used for the microphotographs.

RESULTS

The papain-inhibiting activities of the epidermis and dermis extracts (from a single epidermis-dermis separation) were 0.19 U/ml and 0.12 U/ml respectively, and their protein concentrations 0.75 and 1.88 mg/ml. The total activity of the epidermis extract thus constituted 62% of the combined papain-inhibiting capacity and that of dermis extract 38%. Because of the low protein concentration of the epidermis extract, its specific activity was 4.1 times higher than that of the the dermis extract.

The results of fractionation of the above mentioned extracts on Sephadex G-50 are presented in Fig 1. The epidermis extract contained mainly the low-molecular-weight inhibitor I_2 (fraction No: 50, 1.47 × V_o) and the dermis extract the high-molecular-weight inhibitor I_1 (fraction No: 34, V_o). The ratio I_1:I_2, as calculated from the areas of the inhibitor peaks, was 1:29 in the epidermis extract chromatogram and 8:1 in that of the dermis extract. The results suggest that I_1 is a dermal and I_2 an epidermal inhibitor. The small traces of I_2 in the dermis extract and of I_1 in epidermis extract may be due to cross contamination. Histological examination of the fractions revealed that the dermis was slightly contaminated with epidermal cells, although no dermal tissue was found in the epidermis fraction. Another explanation for the relatively high proportion of I_2 in the dermis extract is that the epithelial appendaces of the

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dermis, i.e., the hair follicles and sebaceous glands, may also contain I_2.

The results of immunodiffusion of I_2, epidermis extract, dermis extract and rat serum against anti-I_2 serum are depicted in Fig 2. Anti-I_2 serum formed a heavy precipitate with I_2 and the epidermis extract, a very weak precipitate with dermis extract (hardly visible in Fig 2), and no precipitate with rat serum. The precipitates with I_2 and epidermis fused at their ends, suggesting that the antigens were identical. The results confirm that I_2 isan epidermal inhibitor.

In order to rule out the possibility that the antibodies obtained by the immunization of rabbits with I_2 may have been specific against some impurity in the antigen preparation, an immunoglobulin fraction was prepared from the anti-I_2 serum and its effect on the papain-inhibiting capacity of I_2 determined (Table). The immunoglobulins did not inhibit papain, but they did destroy the papain-inhibiting activity of I_2, as seen from the decrease in inhibitor activity with increasing immunoglobulin concentrations, the amount of inhibitor remaining constant. The immunoglobulins from normal rabbit serum did not inactivate I_2 or inhibit papain. The result confirms that anti-I_2 serum contains antibodies against I_2.

The localization of I_2 by indirect immunofluorescence was complicated by the high solubility of the inhibitor and its nonresistance to strong fixatives. When frozen sections of rat skin were used, the antigen dissolved immediately when the sections were melted on the microscope slides, giving a cloud of positively fluorescing granules in the epidermis and its vicinity. Attempts to fix the frozen sections with 94% ethanol, acetone, methanol, or Clarce's solution did not improve the localization of I_2, while 1% glutaraldehyde, phosphate buffered 4% formaldehyde, Zenker, and Carnoy fixatives destroyed the antigen. As I_2 is highly resistant to high temperatures [2] and organic solvents, freeze substitution and paraffin embedding of the skin was attempted, as described in "Materials and Methods." In this procedure the skin was dry until the anti-I_2 solution was added (with the exception of the stretching of the sections on the water bath). Using this procedure a bright green fluorescence was seen in the epidermis (Fig 3). The epidermis of a

Effects of immunoglobulins prepared from rabbit anti-I_2 serum on the papain-inhibiting activity of I_2[a]

Immunoglobulins (mg)	I_2 (mg)	Papain inhibition (U/ml)
0.0	0.012	0.077
0.7	0.012	0.040
1.4	0.012	0.018
2.8	0.012	0.009
2.8	0.00	0.00
2.8[b]	0.00	0.00
2.8[b]	0.012	0.078

[a] The inhibitor was preincubated for 20 min at room temperature with various amounts of immunoglobulins in a total volume of 1 ml and the papain-inhibiting activities of the solutions then assayed.
[b] Immunoglobulins from a non-immunized rabbit.

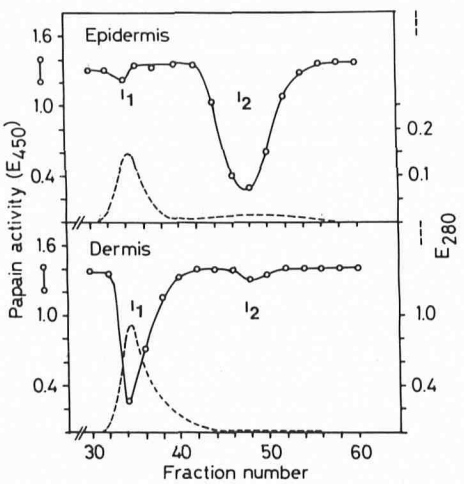

FIG 1. Gel filtration of an epidermis extract and a dermis extract on Sephadex G-50.

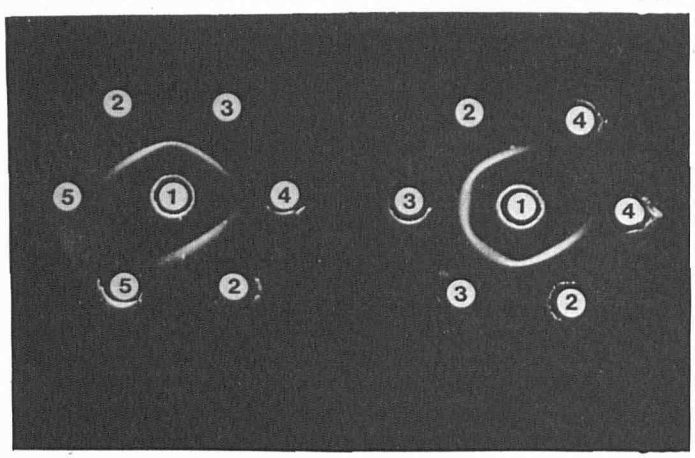

FIG 2. Immunodiffusion. *1* = rabbit anti-I_2 serum (5 μl); *2* = I_2 (0.3 μg); *3* = epidermis extract (5 μl); *4* = dermis extract (5 μl); *5* = rat serum (5 μl).

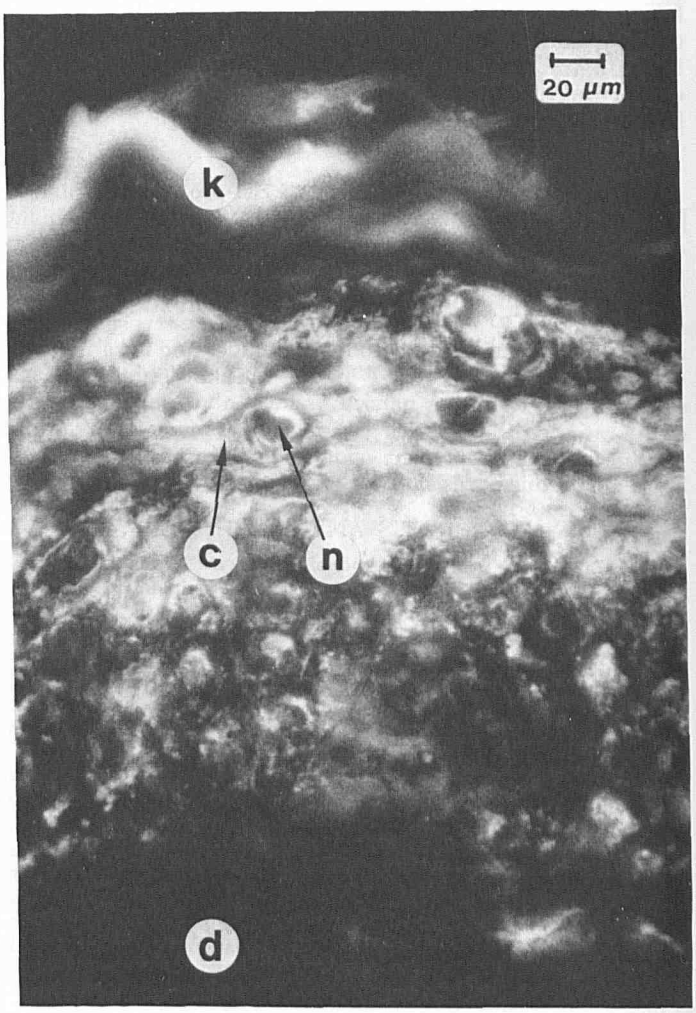

FIG 3. Immunofluorescence of rat epidermis, *k* = keratin layer with yellow autofluorescence. *c* = cytoplasm of an epidermal cell. *n* = nucleus. *d* = dermis. Anti-I_2 serum was diluted with 15 parts of PBS. Magnification in microphotography was 89-fold.

control section, on which normal rabbit serum was applied instead of anti-I_2 serum, was only feebly fluorescent (Fig 4). The keratin layer of the skin had a bright yellow autofluorescence and the hairs an intense nonspecific, green fluorescence, also visible in the control samples. A nonspecific green fluorescence was also seen in the dermal fibroblasts and their extensions between collagen bundles, and in mast cells. The positive fluorescence was most intense in the granular layer of the epidermis. Even though the cellular localization of I_2 was hampered somewhat by improper fixation of the antigen, most of the fluorescence could be localized in the cytoplasm of the epidermal cells. The nuclear membranes also exhibited a bright green fluorescence and the nuclei were seen as dark holes. The positive fluorescence of the epidermis was lost when the antibody solution was absorbed with I_2. The immunofluorescence experiments confirm that I_2 is an epidermal inhibitor.

DISCUSSION

The biochemical, immunological and immunohistochemical experiments presented here clearly indicate that the low-molecular-weight SH-protease inhibitor I_2 in rat skin is epidermal, and it is probably one of the antigens of the rat epidermis described by Räsänen [6]. Evidence is also presented that the high-molecular-weight inhibitor I_1 is dermal. It has previously been suggested that I_1 is derived from serum, which contains large amounts of an inhibitor of similar molecular size [2]. Since I_2 is more active in rat skin than in other tissues and is located in the epidermis, the name epidermal SH-protease inhibitor is suggested for it.

FIG 4. Control immunofluorescence. Non-immune rabbit serum, diluted with 7 parts of PBS was used instead of anti-I_2 serum.

The rat skin epidermal SH-protease inhibitor has properties very similar to those of the inhibitor described by Hayashi et al in Arthus lesions in rabbit skin [7–9], and in rabbit burns [10]. The activity of the Arthus inhibitor increases simultaneously with a decrease in the activity of an SH-dependent inflammatory protease of Arthus lesions in the healing phases of the reaction [7,9]. Cultured mononuclear cells from rabbit peritoneum are seen to synthesize this inhibitor [11], and it has been suggested that mononuclear cells secrete the inhibitor in healing Arthus inflammation [9].

The epidermal localization of the SH-protease inhibitor of normal rat skin suggests that this inhibitor does not participate in Arthus inflammation, which mainly is a dermal reaction. It is not known however, whether the Arthus inhibitor is identical with the epidermal SH-protease inhibitor. Recent experiments with the Arthus reaction in the guinea pig suggest that they are possibly not identical, since the guinea pig inhibitor is probably derived from serum [12], while the antiserum against the rat epidermal SH-protease inhibitor does not precipitate any serum protein in immunodiffusion. Confirmation of the identity or nonidentity of the epidermal inhibitor and Arthus inhibitor would require careful study of the epidermal and dermal components of Arthus reaction sites.

The role of the SH-protease inhibitor in the normal rat epidermis is not known. Its localization in the granular layer of the epidermis suggest that it may be active in the keratinization process in the skin. The inhibitor may also act as a defence mechanism against infection by neutralizing the harmful SH-activated exoproteases of microorganisms.

The epidermis is evidently a better starting material for purifying the inhibitor than is the whole skin. Human skin contains very small amounts of the SH-protease inhibitor [2], but it can certainly be purified by starting with the epidermis, a task which is now in progress in this laboratory.

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